USEPA REGION 9 LABORATORY
RICHMOND, CALIFORNIA

STANDARD OPERATING PROCEDURE 1001
SEA URCHIN (*Strongylocentrotus purpuratus*) AND SAND DOLLAR (*Dendraster excentricus*) FERTILIZATION TOXICITY TEST

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1 SCOPE AND APPLICABILITY

1.1 This method estimates the chronic toxicity of environmental samples (e.g. effluents, receiving waters) to the gametes of sea urchins, (*Strongylocentrotus purpuratus*), or sand dollars (*Dendraster excentricus*). The test measures fertilizing capacity of the sperm with a 20-minute sperm exposure to the sample and a subsequent 20-minute exposure period following the addition of eggs.

1.2 The purpose of the test is to determine the concentrations of a test substance that reduce egg fertilization by exposed sperm relative to that attained by sperm in control solutions. Concentrations of materials adversely affecting egg fertilization under the conditions of this test are usually acutely and chronically toxic to one or more of several common marine test species and, by extension, are presumably acutely and chronically toxic to other of the many untested marine species.

1.3 The procedure follows methods specified in Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms, EPA/600/R-95/136 (USEPA, 1995). U.S. EPA Region 9 Laboratory staff perform toxicity tests on samples from NPDES facilities or Superfund sites.

1.4 Refer to Appendix A for a description of deviations from the reference method. Refer to Appendix B for specific analytes/endpoints. No quantitation limit is established for this method.

2 METHOD SUMMARY

This method measures the toxicity of a sample to sea urchin or sand dollar sperm by exposing known sperm suspensions to various dilutions of the sample for 20 minutes. Known densities of eggs are then added to the exposed sperm to allow the eggs to fertilize for 20 minutes. The test is terminated by the addition of a preservative and the percent fertilization is determined by microscopic examination of 100 eggs in an aliquot of eggs from each treatment. The test endpoint is normal egg fertilization shown by the presence of a fertilization membrane around the egg. Point estimates and hypothesis-derived endpoints are calculated using the CETIS data analysis program (Tidepool Scientific Software, 2001) to determine the samples in which fertilization is reduced or statistically significantly different from the control. The Test of Significant Toxicity (TST) endpoint (USEPA, 2010) is calculated using the TST Calculator to determine whether the critical concentration is toxic when compared to the control (with a "pass" or "fail" outcome).

A positive control or reference toxicant test is conducted with copper chloride. This test is used to provide (A) a measure of acceptability of the test by providing evidence of the health and relative quality of the test organisms, and the suitability of the overlying water, test conditions and handling procedures, etc., and (B) the basis for interpreting data obtained

from the test solutions.

3 DEFINITIONS

A list of terms and definitions specific to this procedure appears below. For terms and acronyms in general use at the EPA Region 9 Laboratory refer to Appendix A of the Laboratory Quality Assurance Plan.

Dilution seawater - Seawater that is essentially free of contaminants and filtered through at least a 1 µm filter. This can be seawater that is for mixing reference toxicant solutions; or as control water, for running treatments without any concentrations of test materials that may cause changes in the larval development of the test organisms.

Effect Concentration (EC50) - The statistically or graphically derived best estimate of the concentration of test material that is expected to cause a measured sublethal effect in 50% of the test organisms under specified conditions.

No Observed Effect Concentration (NOEC) - The highest concentration of test material that does not cause a statistically significant effect on the test organisms under the specified conditions.

Percent Minimum Significant Difference (PMSD) - A measure of the within-test variability representing the amount of difference from the control that can be detected statistically (%MSD = MSD/control mean x 100).

Reference Toxicity Test or positive control test - A test conducted concurrently with a test on environmental samples to determine possible changes in condition of the test organisms and demonstrate a laboratory's ability to obtain consistent results with the test method. Reference toxicity tests are performed with known chemicals in dilution seawater.

Test of Significant Toxicity (TST) - Refers to the statistical application using hypothesis testing to determine whether a sample's critical concentration (e.g., Instream Waste Concentration) and control differ by an unacceptable amount and is considered toxic (USEPA, 2010).

4 SAFETY & HEALTH

All laboratory operations must follow health and safety requirements outlined in current versions of the EPA Region 9 Laboratory Chemical Hygiene Plan and the EPA Region 9 Laboratory Business Plan. Potential hazards specific to this SOP as well as pollution prevention and waste management requirements are described in the following sections.

4.1 Chemical Hazards

Due to the unknown and potentially hazardous characteristics of samples, all sample handling and preparation should be performed in a well-vented area or, if appropriate, laboratory fume hood.

The toxicity and carcinogenicity of each reagent used in this method may not be fully established. Each chemical should be regarded as a potential health hazard and exposure to them should be minimized by good laboratory practices. Refer to the Material Safety Data Sheets located in Room 118 (library) and the LAN at I:\MSDS IMAGES for additional information.

Samples to be tested, especially those from effluent areas, might contain organisms that can be pathogenic to humans. Special precautions when dealing with these samples might include immunization prior to sampling, use of protective gloves and clothing and use of bacterialcidal soap after working with the samples.

Water samples collected from the field might be contaminated with unknown concentrations of many potentially toxic materials. Any potentially contaminated water samples should be handled in a manner to minimize exposure of lab staff to toxic compounds.

4.2 Equipment and Instruments

Follow the manufacturer's safety instructions whenever performing maintenance or troubleshooting work on equipment or instruments. Unplug the power supply before working on internal instrument components. Use of personal protective equipment may be warranted if physical or chemical hazards are present.

4.3 Pollution Prevention

The Region 9 Laboratory's Environmental Management System (EMS) encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for EMS exist in laboratory operations. The EPA Region 9 Laboratory places EMS as the management option of first choice with regard to environmental management. Whenever feasible, laboratory personnel shall use the EMS to address waste generation. The *EPA Region 9 Laboratory Environmental Management System* provides details regarding efforts to minimize waste.

Minimize waste through the judicious selection of volumes for reagents and standard to prevent the generation of waste due to expiration of excess materials. Reduce the volume of any reagent or standard described in Sections 7.2 so long as good laboratory practices are adhered to regarding the accuracy and precision of the glassware, syringes, and/or analytical balances used to prepare the solution. Reduce the toxicity

of waste by purchasing lower concentration stock standards, lower concentration stock reagents, and solutions to replace neat chemicals whenever possible. However, do not change the concentrations of standards and reagents specifically designated in this SOP.

4.4 Waste Management

The EPA Region 9 Laboratory complies with all applicable rules and regulations in the management of laboratory waste. The laboratory minimizes and controls all releases from hoods and bench operations. All analysts must collect and manage laboratory waste in a manner consistent with EPA Region 9 Laboratory SOP 706 *Laboratory Waste Management Procedure*. Solid and hazardous wastes are disposed of in compliance with hazardous waste identification rules and land disposal restrictions. If additional guidance is needed for new waste streams or changes to existing waste streams, consult with EPA Laboratory Safety, Health, and Environmental Manager (LaSHEM) or designees.

This procedure generates the following waste streams:

Waste Stream Description	Waste Label	Hazard Properties
Glutaraldehyde preserved samples	Hazardous	Toxic
Effluent in seawater	Hazardous	Toxic
Copper stock solution, 50,000 µg/L	Hazardous	Corrosive, toxic
Copper solution in seawater, <56 µg/L	Hazardous	Toxic
Laboratory solid waste (Non-biologically contaminated gloves, paper towels, disposable glassware	Non-Hazardous	Not applicable

5 SAMPLE HANDLING AND PRESERVATION

5.1 Containers and Required Sample Volume

Sample containers should be made of inert materials to prevent contamination, which might result in artifactual changes in toxicity. Glass or HDPE bottles or cubitainers are appropriate for this purpose. Minimum sample volume should be 40 mL. Techniques for sample collection, handling and storage are described in the USEPA, 1995 method manual under Section 8 (pp. 43-48).

5.2 Internal Chain-of-Custody

Verify sample IDs and dates and times of collection against the chain-of-custody form. Notify the Sample Custodian of any discrepancies.

Update the LIMS database internal custody form when sample containers are moved from the designated sample location. Change the container disposition to "active out" and the location to the appropriate room number. At the end of the day, return sample containers to the "Home" locations. Update the LIMS database using the "return to home location" feature and update container disposition to "available in". Verify that your initials are recorded whenever you update the LIMS custody information.

5.3 Preservation Verification

Not applicable since preservation is not required for this method.

5.4 Sample Storage

Water samples for toxicity testing should be chilled to 4°C when collected and shipped on ice (USEPA, 2002). Samples must be stored at >0 and ≤ 6 °C in the dark in the refrigerators designated for sample storage in Room 503. Retain samples for 60 days after the final analytical report is sent to the data user.

5.5 Holding Time

Samples should be analyzed within 36 hours. In no case should samples be held for longer than 72 hours and used in a test. Samples may be held up to 72 hours only if the NPDES permitting authority allows an extension in sample holding time.

6 INTERFERENCES

6.1 Results of chronic effluent tests will depend, in part, on temperature, salinity, pH, ammonia, and dissolved oxygen. Factors potentially affecting results from static effluent toxicity tests might include:

6.1.1 Testing of materials at temperatures or salinities other than those at which they are originally sampled might affect contaminant solubility, partitioning co-efficiency, and other physical and chemical characteristics.

6.1.2 Toxic substances can be introduced by dilution water, glassware, hardware, and testing equipment.

6.1.3 Maintaining the integrity of the water sample during its removal, transportation, and testing in the laboratory. Any disruption of the sample complicates interpretations of treatment effects, causative factors and *in situ* comparisons.

7 APPARATUS AND MATERIALS

This section describes recommended apparatus and materials to be used for the analysis. All equipment, reagents, standards, and supplies must meet the technical and QC requirements

of the reference method. Substitutions may be made provided that they are documented and equivalency is maintained.

7.1 Instruments and Equipment

7.1.1 Tanks, trays, or aquaria -- for holding and acclimating adult abalone, e.g., standard salt water aquarium, with appropriate filtration and aeration system.

7.1.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

7.1.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (12°C) prior to the test.

7.1.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

7.1.5 Refractometer -- for determining salinity.

7.1.6 Glass or electronic thermometer -- for measuring water temperature.

7.1.7 pH meter -- for routine physical and chemical measurements.

7.1.8 Dissolved oxygen meter -- for routine chemical measurements.

7.1.9 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

7.1.10 Continuous thermograph -- for measuring environmental chamber temperature.

7.1.11 Fume hood -- to protect the analyst from effluent or glutaraldehyde fumes.

7.1.12 Glass stirring rods -- for mixing test solutions.

7.1.13 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

7.1.14 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

7.1.15 Volumetric pipets -- Class A, borosilicate glass, 1 to 100 mL.

7.1.16 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

7.1.17 Pipet bulbs and fillers -- PROPIPET^{R} or equivalent.

7.1.18 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

7.1.19 Wash bottles -- for dilution seawater, for hydrating sea urchins and sand dollars during spawning.

7.1.20 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

7.1.21 Cubitainers for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labelled and not used for other purposes. Strong solutions of NaOH and formaldehyde should not be held for several month periods in cubitainers: interaction or leaching into solutions of 0.1 N or 1 N NaOH used for pH adjustment of dilution water has caused poor egg fertilization; formaldehyde similarly stored has induced aberrant partial membrane elevation in eggs.

7.1.22 Beakers, 5-10 mL borosilicate glass -- for collecting sperm from sand dollars.

7.1.23 Beakers, 50-100 mL borosilicate glass -- for spawning; to support sea urchins and to collect sea urchin and sand dollar eggs.

7.1.24 Blood cell counter vials-20-mL -- for spawning; to support very small sea urchins and to collect sea urchin and sand dollar eggs.

7.1.25 Beakers, 1,000 mL borosilicate glass -- for rinsing and settling sea urchin eggs.

7.1.26 Vortex mixer -- to mix sea urchin semen in tubes prior to sampling.

7.1.27 Compound microscope -- for examining gametes, counting sperm cells (200-400x) and eggs (100x).

7.1.28 Inverted microscope -- for examining fertilized eggs.

7.1.29 Counter, two unit, 0-999 -- for recording sperm and egg counts.

7.1.30 Sedgwick-Rafter counting chambers -- for counting egg stock and examining eggs for fertilization at the end of the test.

7.1.31 Hemacytometers, Neubauer -- for counting sperm.

7.1.32 Coverslips -- for hemacytometers and counting chambers.

7.1.33 Microscope well slides -- for pre-test assessment of sperm activity and egg condition.

7.1.34 Siphon hose (3 mm i.d.) -- for removing wash water from settled eggs.

7.1.35 Conical centrifuge tubes -- for holding semen.

7.1.36 Perforated plunger -- for maintaining homogeneous distribution of eggs during sampling and distribution to test containers.

7.1.37 60 µm NITEX filter -- for filtering receiving water.

7.2 Reagents and Supplies

Reagents may contain impurities that affect analytical data. Only materials that conform to the American Chemical Society (ACS) specifications should be used. If the purity of a reagent is in question, analyze for contamination prior to use.

Record all chemical and reagent preparations in the LIMS.

7.2.1 Deionized water, laboratory grade 18 megohm -- for reference toxicant preparation.

7.2.2 Tape, colored -- for labelling test chambers and containers.

7.2.3 Markers, water-proof -- for marking containers, etc.

7.2.4 Parafilm -- to cover graduated cylinders and vessels containing gametes.

7.2.5 Gloves, disposable -- for personal protection from contamination.

7.2.6 Pipets, serological -- l-l0 mL, graduated.

7.2.7 Pipet tips -- for automatic pipets. Note: pipet tips for handling semen should be cut off to produce an opening about 1 mm in diameter; pipet tips for handling eggs should be cut off to produce an opening about 2 mm in diameter. This is necessary to provide smooth flow of the viscous semen, accurate sampling of eggs, and to prevent injury to eggs passing through a restricted opening. A clean razor blade can be used to trim pipet tips.

7.2.8 Lens paper -- for cleaning microscope optics.

7.2.9 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

7.2.10 Disposable countertop covering (Kaydry^{R}) -- for protection of work surfaces and minimizing spills and contamination.

7.2.11 Test chambers -- borosilicate glass test tubes, 16 x 125 mm, with caps for conducting the test, four chambers per concentration. All test containers should be soaked in seawater at least 24 hours and triple rinsed in Nanopure deionized water (18 megaohm).

7.2.12 Scintillation vials, 20 mL -- for transferring test tube contents so that test results can be read on an inverted microscope.

7.2.13 Glutaraldehyde, analytical reagent grade,1% in seawater -- for preserving eggs.

7.2.14 Acetic acid, 10%, analytical reagent grade, in dilution seawater -- for preparing killed sperm dilutions for sperm counts.

7.2.15 Haemo-Sol or equivalent cleaner -- for cleaning hemacytometer and cover slips.

7.2.16 0.5 M KCl solution, analytical reagent grade -- for inducing spawning.

7.2.17 Syringe, disposable, 3 or 5 mL -- for injecting KCl into sea urchins and sand dollars to induce spawning.

7.2.18 Needles, 25 gauge -- for injecting KCl.

7.2.19 Disposable pipets -- for sampling eggs from spawning beakers.

7.2.20 Hematocrit capillary tubes -- for sampling sperm for examination and for loading hemacytometers.

7.2.21 $\leq$ 1 µm filtered natural seawater -- dilution water for control treatments and reference toxicant solutions.

7.2.22 Copper chloride ($CuCl_2 \cdot 2H_20$), analytical reagent grade -- for reference toxicant preparation.

8 ANALYTICAL PROCEDURES

8.1 Effluent Preparation

8.1.1 Most NPDES permits specify the dilution series recommended for effluent toxicity tests, which normally include the instream waste concentration (IWC), two dilutions above the IWC, and two dilutions below the IWC. The IWC is the concentration of effluent at the edge of the mixing zone.

8.1.2 If the permit specifies a TST approach, then the instream waste concentration may be compared to a control concentration. In this case, the number of replicates can be increased (e.g., 10 replicates)

8.1.3 For test solutions at low effluent concentrations (<6%), add effluents directly to the dilution seawater. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. See Table 8.1 for an example of effluent solutions. Fill the volumetric flask to the 100-mL mark with dilution seawater, stopper it, and shake to mix. Record all effluent dilutions in Worksheet 8 (Appendix M) or Worksheet 9 (Appendix N).

8.1.4 To prepare test solutions at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine and top off the flask with dilution water. Stopper the flask and shake well. See Section 8.2.2 for the equation used to determine brine solutions.

8.1.5 Number each test container with the random number generated in CETIS. Pour 5 mL of each test solution into a minimum of 4 replicate test containers for each concentration. The remaining test solutions can be used for chemistry and water quality measurements. Randomize the placement of test chambers by placing them in numerical order in the environmental chamber (since each test container has already received a random number from CETIS).

8.1.6 If an inverted microscope will be used to count fertilized eggs, test tube contents should be transferred to scintillation vials after the test is complete. If a compound microscope and counting chambers will be used for counting fertilized eggs, seawater-soaked test tubes can be used as test containers.

Table 8.1 Example of effluent preparation for the sea urchin fertilization toxicity tests.

EFFLUENT CONCENTRATION (%)	VOLUME EFFLUENT (mL)	TOTAL VOLUME SEAWATER (mL)
0	0	100
0.15	0.15	100
0.30	0.30	100
0.60	0.60	100
0.12	0.12	100
0.24	0.24	100

8.2 Control and Blank Treatments

8.2.1 Control treatments (0% effluent) must consist of at least 4 replicates of dilution seawater ($\leq$ 1 µm filtered natural seawater).

8.2.2 Brine controls must be used in all tests where hypersaline brine is used to increase sample salinity. Hypersaline brine controls should be made by adding the same volume of brine as the highest sample concentration using brine, plus the volume of deionized water needed to reproduce the hyposalinity of the sample in the highest concentration, plus dilution seawater. Calculate the amount of deionized water to add to the brine controls by using the following equation:

$$VD = VB \times (SB - 34)/(34 - SD)$$

VD = volume of the deionized water add in mL
SB = salinity of the brine in ppt
SD = salinity of deionized water in ppt (0 ppt)
VB = volume of brine in mL

8.2.3 Brine controls must be used in all tests where modified GP2 salts are used to increase sample salinity. Prepare a modified GP2 brine control by diluting the dilution sea water to the salinity of the sample prior to any manipulations. Add the same amount of dry GP2 salts to the brine control as the weight of GP2 salts that were added to the sample.

8.3 Egg Blanks

8.3.1 Place two replicates of effluent egg blanks at the beginning of the gamete injection sequence containing 5 mL of the highest sample concentration. Cap these blanks during sperm addition and add only eggs to these test containers to determine whether the sample induces a false fertilization membrane.

8.3.2 Position two replicates of egg blanks at the end of the gamete injection sequence containing 5 mL of dilution seawater. Cap these blanks during sperm addition and add only eggs to these test containers to determine whether accidentally fertilized eggs were used in the test.

8.4 Sperm Density Controls

8.4.1 Regardless of the sperm density chosen for the definitive test, sperm density controls are required.

8.4.2 Fill two replicates of low sperm density controls with 5 mL of dilution seawater. These sperm controls will receive 0.05 mL of sperm stock (half of the required 0.10 mL sperm stock) in addition to the normal 0.5 mL egg stock.

8.4.3 Fill two replicates of high sperm density controls with 5 mL of dilution seawater. These sperm controls will receive 0.20 mL of sperm stock (twice the required 0.10 mL sperm stock) in addition to the normal 0.5 mL egg stock.

8.4.4 Control fertilization in the 0.05 mL sperm stock control must be 5% lower than the 0.2 mL controls. Calculate the difference using Worksheet 7, Appendix L.

8.5 Reference Toxicant Preparation

8.5.1 Conduct reference toxicity tests concurrently with toxicity tests on environmental samples to determine changes in test organism condition and sensitivity.

8.5.2 Prepare a copper stock solution of 50,000 µg/L by weighing 0.0134g $CuCl_2 \cdot 2H_2O$ on an analytical balance, adding it to a 100-mL volumetric flask, bringing the flask to volume with deionized water, and stirring until the solid is dissolved.

8.5.3 Number each test container with the random number generated in CETIS. At least five concentrations (0.5 dilution factor) of copper chloride should be tested, in addition to a control of seawater only. At least four replicates of each concentration must be used. Pour 5 mL of reference toxicant into each of 4 replicates of test containers. Randomize the placement of test chambers by placing them in numerical order in the environmental chamber (since each test container has already received a random number from CETIS).

8.5.4 Prepare dilutions of copper chloride in the following copper concentrations (Table 8.2) and record reference toxicant preparations in the Prepared Standard and Reagent Log in Rm. 308. Record dilutions of reference toxicant and stock solution preparation in the Effluent and Reference Toxicant Preparation Worksheet 8 or 9 (Appendix M or N).

8.5.5 If an inverted microscope will be used to count fertilized eggs, test tube contents should be transferred to scintillation vials after the test is complete. If

a compound microscope and counting chambers will be used for counting fertilized eggs, seawater-soaked test tubes can be used as test containers.

Table 8.2. Reference toxicant preparation for echinoderm fertilization toxicity tests.

COPPER CONCENTRATION (µg/L)	VOLUME STOCK COPPER SOLUTION (µL)	TOTAL VOLUME SEAWATER (mL)
0	0	100
5.6	11.2	100
10.0	20.0	100
18.0	36.0	100
32.0	64.0	100
56.0	112.0	100

8.6 Test Species, Sea Urchins

8.6.1 Sea urchins should be collected, shipped, acclimated and held according to Section 16.6.32 in EPA/600/R-95/136 (USEPA, 1995).

8.6.2 Collect adult sea urchins (*Strongylocentrotus purpuratus*) from uncontaminated intertidal or subtidal areas. Animals can be collected in the intertidal region of Garrapata State Park south of Carmel Highlands since spawning season appears to be year-round in this area.

8.6.3 Transport sea urchins in coolers surrounded by seawater-soaked paper towels on top of insulated ice packs to prevent thermal shock and premature spawning.

8.6.4 Maintain adult sea urchins in spawning condition year-round in Rm. 306 (Culture Room) at a constant temperature (15 ^{o}C) in darkness at a salinity between 32-36 ppt. Once sea urchins have been spawned, they can be separated by sex in aquaria for future tests. Feed urchins blades of rehydrated *Macrocystis* (giant kelp) every other day.

8.1.5 At least 24 hours prior to test initiation, record urchin mortality, disease, or stress (only if any of these conditions exists) in the Aquarium Test Organism Log.

8.7 Test Species, Sand Dollars

8.7.1 Sand dollars should be collected, shipped, acclimated and held according to Section 16.6.33 in EPA/600/R-95/136 (USEPA, 1995).

8.7.2 Collect adult sand dollars (*Dendraster excentricus*) from uncontaminated subtidal areas. Sand dollars can also be obtained from commercial suppliers.

8.7.3 Transport sand dollars in coolers surrounded by seawater-soaked paper towels on top of the insulated ice packs to prevent thermal shock and premature spawning.

8.7.4 Hold sand dollars between 8-12 °C at a salinity between 32-36 ppt. Once sand dollars have been spawned, they should be discarded since they do not maintain spawning condition over long periods of time.

8.7.5 At least 24 hours prior to test initiation, record sand dollar mortality, disease, or stress (only if any of these conditions exists) in the Aquarium Test Organism Log.

8.8 Collection of Gametes for the Test

8.8.1 Spawning Induction

8.8.1.1 Pour 12°C filtered seawater into spawning beakers in 12°C environmental chamber. Select a sufficient number of sea urchins or sand dollars (based upon recent or past spawning success) so that three of each sex is likely to provide gametes of acceptable quantity and quality for the test. At least 6 of each sex (if known) is appropriate.

8.8.1.2 Remove sea urchins or sand dollars from holding tanks and place into a container lined with seawater-moistened paper towels to prevent reattachment.

8.8.1.3 Handle sexes separately once known; this minimizes the chance of accidental egg fertilization. Throughout the test process, it is best if a different worker, different pipets, etc. are used for males (semen) and females (eggs). Frequent washing of hands is a good practice.

8.8.1.4 Fill a 3-5 mL syringe with 0.5 M KCl and inject 0.5 mL through the soft peristomial membrane of each sea urchin or the oral opening of each sand dollar. Gently swirl sea urchins in a circular, horizontal motion for several seconds. Place the sea urchins onto spawning beakers oral side up and sand dollars oral side down. If sexes are known, use a separate needle for each sex. If sexes are not known, rinse the needle with alcohol between each injection to avoid the accidental injection of sperm from males into females.

8.8.1.5 Note time of spawning and sex of each individual on Worksheet 1, Appendix F. Do not collect gametes from any individual for more

than 30 minutes after the first injection. Turn spawning sea urchin males oral side down on a seawater-moistened paper towel for semen collection. This ensures that sperm are only collected "dry" from the males as the gametes are shed over the top surface of the individual and not into the dilution water. Turn the spawning sand dollar males oral side up on 5-10 mL glass beakers filled with 12°C seawater. Leave female sea urchins and sand dollars to shed eggs oral side up into the 50 or 100-mL beakers. Sea urchin females will release clear or orange eggs and sand dollar females will release purple eggs. Males will release cream-colored semen.

8.8.1.6 Note time of spawning and sex of each individual on Worksheet 1 (Appendix F). Do not collect gametes from any individual for more than 30 minutes after the first injection.

8.8.1.7 If spawning does not occur after 5 or 10 minutes, try injecting the urchin with a second 0.5 ml injection. Do not inject more than 1.0 ml of potassium chloride into one urchin since it seldom results in good quality gametes and may result in mortality of the test organisms.

8.8.2 Collection and Storage of Sperm

8.8.2.1 Collect semen from male sea urchins dry (directly from the surface of the sea urchin), using a Pasteur pipet. Pipet semen from each male into separate 3 mL labeled conical test tubes, store in an ice water bath or refrigerator (<5°C) and cover with parafilm.

8.8.2.2 Collect sperm from male sand dollars after decanting the overlying seawater.

8.8.2.3 The sperm should be used in a toxicity test within 4 hours of collection.

8.8.3 Viability and Pooling of Sperm

8.8.3.1 Early in the spawning process, place a very small amount of sperm from each male into dilution water on a well microscope slide. Examine the sperm for motility and make note of motility on Worksheet 1 (Appendix F). Only use sperm from male(s) with high sperm motility.

8.8.3.2 At least 0.5 mL of pooled semen should be available for the test. If this cannot be collected from one male, pool equal quantities of semen from each of the sea urchin or sand dollar males that has been deemed good.

8.9 Preparation of Egg Suspension for Test

8.9.1 Acceptability and Pooling of Eggs

8.9.1.1 A small sample of the eggs from each female should be examined for the presence of significant quantities of poor eggs (vacuolated, small, or irregularly shaped). The egg sample should be mixed with good sperm to determine the extent of fertilization. Note the condition of the eggs and successful fertilization on Worksheet 1 (Appendix F). If good quality eggs are available from one female, pooling of eggs is not necessary.

8.9.1.2 Pool the sea urchin eggs into a 1 L beaker, and bring the volume to 600 mL with 12°C dilution water. Swirl the eggs into suspension and allow them to settle for 15 minutes at 12°C. Siphon off 500 mL of the overlying water with airline tubing (along with any feces or spines), and bring the volume back to 600 mL with more 12°C dilution seawater.

8.9.1.3 Resuspend the eggs again and allow them to settle for a second 15 minute period. After again siphoning off the overlying 500 mL, gently pour the rinsed eggs into a 100 or a 250 mL graduated cylinder and bring to volume with 12°C dilution water. Eggs are stored at 12°C throughout the pre-test period. NOTE: The egg suspension may be prepared during the 20 minute sperm exposure.

8.9.1.4 Pool sand dollar eggs if necessary and do not rinse due to their fragility. Mix eggs once by gently inverting them in a graduated cylinder before sub-sampling for egg stock calculations.

8.9.2 Density of Eggs

8.9.2.1 Place 9 mL of dilution water in each of two 22 mL liquid scintillation vials labeled A and B. Mix egg stock well with an egg plunger or by inversion and place 1 mL into vial A. Transfer 1 mL of well-mixed egg suspension from vial A into vial B. Transfer 1 mL of well-mixed egg suspension from vial B to a Sedgewick-Rafter counting chamber. Count eggs from vial B on a compound microscope at 40x or 100x magnification. Maintain the egg stock by covering with parafilm and store at 12°C.

8.9.2.2 Prepare 100 mL of egg stock in dilution water at the final target concentration of 2,240 eggs/mL (224,000 eggs in 100 mL) by using the Worksheet 2 (Appendix G).

8.9.2.3 Check the egg stock density. Add 1 mL of the final egg stock to 9 mL of dilution water in a scintillation vial. Mix well and transfer 1 mL into a Sedgewick-Rafter counting chamber. The egg count should be 200-245 in the dilution (2000-2450 eggs/mL in the final stock). Adjust egg stock volume and recheck counts if necessary to obtain counts within this range.

8.10 Acceptable Sperm Density

8.10.1 To reduce the likelihood of a failed test due to inadequate control fertilization or exceeding the maximum acceptable sperm density, use one of the two approaches:

8.10.1.1 Conduct the test at a low sperm density in which over-sperming does not create test insensitivity. This can be met by using a confirmed sperm stock density of $\leq 5.6 \times 10^6$/mL (this is equivalent to a sperm:egg ratio of $\leq$ 500:1 at 2240 eggs/mL); OR

8.10.1.2 Conduct a fertilization trial test to determine the sperm density that will provide about 80-100 percent control egg fertilization and avoid significant "oversperming" that can reduce test sensitivity; Section 8.13.

8.10.2 A definitive toxicity test cannot be completed if the sperm:egg ratio exceeds 3000:1. This is a cut-off based on gradual loss of test sensitivity at higher sperm densities, even in cases where control fertilization is considerably below 100 percent.

8.11 Preparation of Sperm Dilution for Use in the Trial for Estimating Appropriate Sperm Density for Definitive Test

8.11.1 For sea urchins and sand dollars, prepare a killed sperm preparation to determine the dilution required to obtain a sperm stock at a concentration for the maximum sperm density needed for the trial or definitive test. A spectrophotometric or microscopic measurement can be used.

8.11.2 Spectrophotometric measurement of sperm

8.11.3 A rapid measurement using a spectrophotometer may be used to determine initial sperm density. A regression equation developed from a correlation between microscope counts and absorbance readings of sperm samples is used to determine the sperm counts (Appendix P).

8.11.4 Warm up the spectrophotometer for 30 minutes. Use Worksheets 3 and 4 (Appendices H & I) to record the following measurements and calculate sperm densities. Mix the pooled semen (8.8.10 or 8.8.11) by agitating the centrifuge tube for about 5 seconds using a vortex mixer. Withdraw a sample of semen using a Pasteur pipet and empty pipet contents into a scintillation vial that is tared on a balance. Add approximately 0.05 g of the concentrated sperm. Note the initial sperm weight (S1). Dilute to about 10g with dilution seawater and note the final weight (W1). Cap the vial and mix the contents.

8.11.5 Into a second tared scintillation vial, add between 1-2 g of the first dilution, and note the weight (S2). Dilute to about 10 g with dilution seawater and note the final weight (W2).

8.11.6 Read the absorbance of the diluted sperm (vial 2) in a 1-5 cm cuvette at 750 nm using filtered (dilution) seawater as a blank.

8.11.7 Calculate the sperm cell density of the diluted sperm from the regression equation using Worksheet 4 (Appendix I) and the equations below:

$Y = [b_o + b_1 x_i]$
where Y is the diluted sperm concentration (sperm/mL);
b_o is Y-intercept
b_1 is the regression coefficient (slope)
x_i is the absorbance reading

To determine the density of the pooled sea urchin semen, multiply the diluted sperm density by the dilution factor:

$$\text{Sperm/mL in pooled stock (SPM)} = Y * \frac{W1 \times W2}{S1 \times S2}$$

where Y = sperm/mL in diluted sperm solution and

$\frac{W1 \times W2}{S1 \times S2}$ is derived from weights in 8.11.4 & 8.11.5

8.12 Microscopic measurement of sperm

8.12.1 Mix the pooled semen by agitating the centrifuge tube for about 5 seconds using a vortex mixer. Very slowly withdraw a 0.025 mL subsample of semen using an automatic pipet, wipe off the outside of the pipet tip with tissue, and empty the pipet contents into a vial containing a 100 mL solution of 1% glacial acetic acid in dilution water (e.g., 10 mL of 10% glacial acetic acid plus 90 mL of dilution water).

8.12.2 Repeatedly rinse the residual semen from the pipet tip by filling and emptying until no further cloudy solution is discharged from the pipet tip (this requires several dozen rinses). Cover the vial with parafilm and mix thoroughly by repeated inversion. Note: to obtain quantitatively repeatable samples of semen it is important that: (1) the pipet tip have an opening of at least 1 mm; (2) samples be withdrawn slowly to avoid cavitation and entrainment of air in the semen sample; (3) samples not include fragments of broken spines (which usually settle to the test tube bottom upon vortexing); and (4) wiping semen from the pipet tip with tissue be done with care to avoid wicking semen from within the pipet tip.

8.12.3 Using a hematocrit tube, transfer a sample of the well-mixed preserved sperm suspension to both sides of two Neubauer hemacytometers. Let the sperm settle 15 minutes.

8.12.4 Count the sperm under the microscope at 400x in the first large square. If the number is 20 or greater, count each side of the hemacytometer by reading all 4

perimeter large squares and the center large square (80 small squares total). If the nuber is less than 10, count all 25 squares. If the number is between 10 and 19, count 9 large square in a diagonal patter of the left and right.

8.12.5 When making sperm counts, scan small squares starting from the upper left to right in the top row and right to left in the next row. To avoid counting sperm twice, count all sperm touching the top and left boundary lines, but none on the lower and right lines in each small square

8.12.6 Record all hemacytometer counts on Worksheet 5 (Appendix J). After reading the grids on each side of the hemacytometer, determine whether a second hemacytometer should be read. If the lower count is at least 80% of the higher count, use the mean count to estimate sperm density in semen and the required dilution volume for the test stock. If the two counts do not agree within 20%, count the two fields on the other hemacytometer.

8.12.7 Calculate the sperm density in the semen using the mean of all four counts unless one count can be eliminated as an obvious outlier.

8.12.8 Determine the density of sperm in the pooled stock using Worksheet 5 (Appendix J).

8.13 Dilution of sperm stock

8.13.1 Determine the volume of seawater needed to dilute a sperm stock of 0.025 mL to achieve the sperm density required for a 3000:1 sperm/egg ratio for the final test or trial using Table 1 (Appendix Q).

8.13.2 If the sperm density needed is only a fraction of 3000:1, then multiply the value determined in 8.10.1 by the fraction of 3000. For example, if 500:1 is the ideal sperm:egg (S:E) ratio, multiply the value determined in 8.10.1 by 6.

8.13.3 If a sperm density trial is not used, determine the volume of seawater needed to dilute a sperm stock of 0.025 mL to achieve the sperm density required for a 500:1 sperm/egg ratio for the final test or trial using Table 2 (Appendix Q).

8.14 Sperm Density Trial

8.14.1 The series of trial sperm:egg ratios should include 3000:1 and several lower ratios. Combine sperm stock and seawater in a scintillation vial as shown in Table 8.3 for each sperm:egg ratio. Prepare 5 scintillation vials for 1 replicate of each sperm:egg ratio in the sperm density trial.

8.14.2 Remove 5 mL of the seawater/sperm solution from each container and add to the second container labeled with the same S:E ratio. Add 500 µL of 10% acetic acid to each of these replicates and load a sample onto a hemacytometer for counts. It saves time if these can be prepared and loaded onto hemacytometers while the trial is being conducted. Alternatively, once the trial has been evaluated, the selected nominal sperm density can be confirmed by a

direct hemacytometer count or spectrophotometric measurement. Use Worksheet 6 (Appendix K) to determine the sperm density.

8.14.3 A 20-minute sperm "exposure" can be used in the trial, followed by 20-minute fertilization period following egg addition. If a shorter exposure is needed, the sperm exposure should be 5 minutes followed by a 5 minute fertilization period. After the sperm exposure, add 0.5 mL of egg stock (containing 2240 eggs/mL) to each of the original test containers without acetic acid.

Table 8.3 Preparation of sperm:egg ratios in sperm density trial.

S:E Ratio	Sperm Stock (3000:1) Volume (mL)	Volume Seawater (mL)
3000:1	10	0
1288:1	4.3	5.7
550:1	1.8	8.2
234:1	0.8	9.2
100:1	0.3	9.7

8.14.4 The trial is stopped by the addition of a preservative after 5-20 minutes. Add 0.5 mL of 1% glutaraldehyde to each test container. Except for the use of only one replicate, the procedures, volume, duration, etc. are as specified for the normal controls in the definitive test (Section 8.14).

8.14.5 After the addition of preservative, evaluate the sperm density trial by counting 100 eggs from each scintillation vial until a suitable sperm density can be determined for the definitive test. Examples of sperm density selection are given in Table 8.4. Percent fertilization may be lower in the test than in the trial because the viability of the stored sperm may decrease during the period of the trial. If the sperm have very good viability (e.g., cases 1 and 2, Table 8.4), this loss of viability should be small. If viability is inherently poorer (cases 3, 4, and 5, Table 8.4), the loss of viability could be greater and probably should be taken into account in selecting the sperm density for the test. Case 6 (Table 8.4) represents a special case in which egg viability may affect the percent fertilization; in this case the asymptote of the fertilization curve is assumed to represent 100% fertilization for purposes of selection of sperm density for the test.

8.14.6 Record all the counts made in the sperm trial on Worksheet 6 (Appendix K), select a target sperm:egg ratio for the test, and calculate the dilution of the

pooled sperm stock needed to provide the necessary sperm density for the definitive test by using the examples in Section 8.12.

Table 8.4. Examples of results of trial fertilization tests with specified sperm densities and target sperm density selection (sperm:egg ratio) for the definitive test.

Sperm:Egg	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
100:1	100*	95*	85	70	40	70
234:1	100	98	95*	80	64	85*
550:1	100	100	98	98*	82	89
1288:1	100	100	100	100	84	90
3000:1	100	100	100	100	88*	90

* Recommended selection (interpolation to intermediate sperm:egg ratios may be used if found desirable)

1. If all trials exceed 90% fertilization, select 500:1 (case 1 and case 2).

2. If not all trials exceed 90% fertilization, select the lowest sperm:egg ratio that does exceed 90% fertilization (case 3 and case 4).

3. If no trials exceed 90% fertilization, select the highest sperm:egg ratio (case 5) unless fertilization appears to become asymptotic below 100% (case 6).

4. If even the highest sperm:egg ratio fails to achieve 70% fertilization it is probable that an acceptable test cannot be conducted with these gametes.

8.15 Start of the Definitive Test

8.15.1 Sperm Exposure

8.15.1.1 Prepare a fresh dilution of sperm using either $\leq$500:1 S:E ratio or a higher ratio determined in the sperm density trial (Section 8.13). Mix the iced sea urchin semen stock as described in Section 8.11.9 and Section 8.11.10 (do not kill the sperm). Combine the required volumes of sperm and dilution water determined in Section 8.12 and mix this sperm stock well by repeated inversion of the graduated cylinder or beaker. Begin the test within 5 minutes.

8.15.1.2 Into each test chamber, inject 0.100 mL of the sperm stock (except egg blanks and sperm density controls, see Sections 8.3 and 8.4) and note the time of first and last injection. Use caution when injecting sperm to ensure that the entire volume goes directly into the test

solution and not onto the side of the test container. Similarly, the pipet tip should not touch the test solution or the side of the test tube, risking transfer of traces of test solution(s) into the sperm stock. Mix the sperm stock frequently to maintain a homogeneous sperm stock.

8.15.1.3 Using repeated single 0.100 mL refill and injection, about 12 tubes per minute is a reasonable injection rate. Sperm injection rate (tubes/min) should not exceed that possible for egg injection. Record the time of start and finish of sperm addition on Worksheet 10 (Appendix O).

8.15.2 Sperm Density Confirmation

8.15.2.1 Confirm the sperm count by sampling from the sperm test stock. Add 9 mL of test sperm stock to a scintillation vial containing 1 mL of 10% acetic acid. Fill both sides of a hemacytometer with this dilution after mixing well. Let stand for 15 minutes. Count both sides of the hemacytometer using the counting pattern outlined in Section 8.11.12 and use the average count to calculate the sperm/mL and the sperm:egg ratio actually used in the test.

8.15.2.2 Use Worksheet 7 (Appendix L) to calculate the sperm density in the sperm stock and the final sperm:egg ratio. For a sperm:egg ratio of 3000:1 the stock sperm density will be 3.36×10^7 sperm/mL. When counting 5 large squares (see Section 8.11.12), this amounts to a total count average of 101.

8.15.3 Adding Eggs to the Test

8.15.3.1 Exactly 20 minutes after the sperm were added to the first test container, begin to add the eggs, delivering 0.5 mL of egg stock every test chamber (including egg blanks-see Section 8.3). Follow the same pattern of introduction for the eggs as used with the sperm so that each test chamber has a sperm incubation period of 20 minutes. Agitate the egg stock in a beaker using a perforated plunger to achieve uniform distribution of eggs. Gently swirl test tube rack or scintillation vial holder to ensure mixing of eggs and sperm. Note the time of start and finish of egg addition on Worksheet 10 (Appendix O). This duration should be within one minute of that used for the sperm.

8.15.3.2 The eggs should be injected using a pipet with an opening of at least 2 mm in order to avoid damaging the eggs and to provide sufficient flow to obtain a representative sample.

8.16 Water Quality Measurements

8.16.1 Measure dissolved oxygen (SOP #1061), salinity (SOP #1067), temperature and pH (SOP #1060) on test solutions at the beginning of the test. Water

quality measurements should be taken in a minimum of one test container from each treatment or in a separate test container intended for water quality measurements only. The raw data are recorded into the CETIS database, for report and statistical calculations.

8.16.2 Continuously monitor the temperature in a temperature blank (test container containing 5 mL of dilution water) during the test using a temperature chart-recorder or record temperature several times between the beginning and the end of the test.

8.17 Termination of the Test

8.17.1 Sample Preservation

8.17.2 Exactly 20 minutes after the egg addition, stop the test by adding the fixative glutaraldehyde to kill the sperm and eggs and to preserve the eggs for examination. The time allotted to fixative addition should be about the same as that for sperm and egg addition and the sequence of addition the same as for the introduction of the gametes. Record the start and finish time of fixative addition on Worksheet 10 (Appendix O).

8.17.3 Add 1% glutaraldehyde (vol/vol) in clean seawater at the rate of 0.5 mL to each test container. Make 1% glutaraldehyde by diluting 4 mL of 25% glutaraldehyde with 96 mL seawater.

8.17.4 Mix contents of test containers by inversion or shaking after they have been capped.

8.18 Counting Eggs

8.18.1 Conduct egg counts within 48 hours of test termination.

8.18.2 If test tube contents are transferred to scintillation vials, examine 100 eggs in the bottom of each vial using 100x magnification with an inverted microscope. Work across the vial to avoid recounting the same areas and record the number of fertilized eggs and unfertilized eggs using hand counters.

8.18.3 Fertilized eggs contain fertilization membranes or halos around the egg; unfertilized eggs do not contain fertilization membranes. If fertilization membranes are elevated partially or only slightly around the egg, they must still be considered fertilized.

8.18.4 If test tubes were used for test containers, remove most of the overlying liquid from the tube. Resuspend the concentrated eggs by filling and emptying a 1 mL pipet about 5 times transfer 1 mL of the egg suspension into a 1 mL Sedgewick-Rafter counting chamber. Examine 100 eggs using 100x magnification and score for the presence or absence of an elevated fertilization membrane.

8.18.5 Do not count obviously smaller (often denser) eggs, normal sized eggs with a distinct, clear center, and very large eggs with often irregular color and density.

8.18.6 Record all egg counts for each test chamber number in the CETIS datasheets.

8.19 QC Review

8.19.1 Process and review the results in CETIS and/or TST Calculator. Generate a new reference toxicant control chart using the most recent 20 tests. See Section 9.4 for Test Acceptability Criteria and Section 9.5 for Method Performance.

8.19.2 Review all sample results.

8.19.3 Qualify and flag results in the LIMS Data Entry/Review table following Appendix R of the EPA Region 9 Laboratory Quality Assurance Plan.

9 QUALITY CONTROL

The EPA Region 9 Laboratory operates a formal quality control (QC) program. As it relates to this SOP, the QC program consists of a demonstration of laboratory capability, the concurrent analysis of laboratory control solutions and reference toxicants. The laboratory is required to maintain performance records that define the quality of the data that are generated. A summary of QC criteria is provided in Appendix C.

9.1 Demonstration of Capability

A Demonstration of Capability must be in place prior to using an analytical procedure and repeated if there is a change in instrument type, personnel, or method. Follow procedures described in EPA Region 9 Laboratory SOP 885 *Demonstration of Capability – Biology* for more details.

9.2 Quality Assurance

Quality assurance for aquatic toxicity testing must address all the activities which may affect the final data, such as: 1) sampling and handling; 2) the source and condition of the organisms; 3) test conditions; 4) use of reference toxicants; 5) recordkeeping; and 6) data evaluation.

9.3 Test Conditions

The test conditions should meet the following criteria:

9.3.1 Maintain solutions at a constant temperature ($12 \pm 1^{\circ}C$)

9.3.2 Maintain dissolved oxygen at ≥ 4 mg/L dissolved oxygen

9.3.3 Maintain salinity at 34 ± 2 ppt.

9.4 Test Acceptability Criteria

Quality and acceptability of performance of test organisms is assessed as follows:

9.4.1 Mean egg fertilization in the controls must be $\geq$ 70%.

9.4.2 Egg fertilization at the NOEC in the reference toxicant must be greater than 80% of that in the controls.

9.4.3 The percent minimum significant difference (% MSD) in the reference toxicity test is <25% relative to the control.

9.4.4 The sperm count for the final sperm stock must not exceed 33,600,000/mL (3000:1 sperm:egg).

9.4.5 If the sperm count for the final sperm stock is between 5,600,000 and 33,600,000/mL it must not exceed 2x of the target density from the trial, or if no target density was specified for the test, the high sperm density controls (0.2 mL sperm stock) must have at least 5% higher fertilization than the low sperm density controls (0.05 mL sperm stock).

9.4.6 Dilution water egg blanks and sample egg blanks must not contain eggs with fertilization membranes.

9.4.7 A test may be conditionally acceptable if temperature, dissolved oxygen, salinity, temperature, and other specific conditions fall outside of the specified range, depending upon the level of departure and objectives of the tests.

9.4.8 A concurrent reference toxicant test must be performed, and the endpoint of that test must fall within the control limits established for the reference toxicant/organism/exposure regime tested. The control limits are set at $\pm$ 2 standard deviations from the mean EC_{50} response of at least 5 and up to the last 20 reference toxicant tests run in the lab. If the results fall outside the control limits, data must be qualified and the reference toxicity test may be repeated after careful review.

9.5 Method Performance

9.5.1 A number of factors can affect the results of a chronic toxicity test. The most important sources of uncertainty include: the health of the broodstock and test organisms; the temperature of the test; the measurement of sample volumes/concentrations; and the quality of the control dilution water. The quality of each of these potential sources of uncertainty is controlled by various measures in the SOP.

9.5.2 Method performance is assessed using control performance and a reference toxicant sample. The control limits are listed in Appendix C. Per the method, a control chart of reference toxicant response (using an EC_{50}) must be

maintained. A concurrent reference toxicant test is run with each set of samples. The response of the reference toxicant must be within 2 standard deviations of the ongoing central tendency of the most recent 20 tests. The results of any samples associated with a response outside the criteria must be flagged, and possibly reanalyzed.

9.5.3 After preparation, all reference toxicant stock concentrations will be analyzed by the Region 9 Laboratory. If the measured value of reference toxicant metal is within 15% of the nominal value, the analyst will not recalculate the reference toxicant concentrations. If the measured value of the reference toxicant is outside of 15% of the nominal value, the analyst will recalculate the reference toxicant concentrations, re-analyze the data using statistical analyses, and re-plot the data in the control chart.

10 DOCUMENTATION

10.1 Each reagent and standard that is used or prepared for the analysis is entered into the LIMS. A label is created for each standard and the unique identification number is used to track the standard and its use.

10.2 The raw data will be entered into the CETIS statistical program. Raw data includes the total number of eggs counted and the number of fertilized eggs, as well as routine chemistry information. The CETIS database program will maintain an electronic copy of all the raw data as well as perform the statistical analyses following the flowchart of statistical analyses that are recommended in the method manual USEPA, 1995 (p. 62, Figure 2). The Percent Minimum Significant Difference (PMSD) will be calculated for each reference toxicity test.

10.3 Test of Significant Difference: If the permit or project requires a Test of Significant Difference (TST), then the raw data is entered into the TST Calculator program. Raw data includes the total number of organisms counted and the response (number of fertilized eggs) for both the control and the critical concentration. The TST Calculator performs statistical analysis and produces either a pass or fail (non-toxic or toxic) response.

10.4 A hard copy of the database raw data, the routine water chemistry data, and the results of the data analysis including EC_{50}s, ICPs, and NOECs will be created. The reference toxicant test results will be entered and analyzed by the database system. A reference toxicant control chart with the concurrent test and 19 previous reference toxicity tests will be created.

10.5 The endpoint results will be inputted into the LIMS system. A data package will be created which contains at a minimum the following: a case narrative; a summary of the raw data including the routine water chemistry data; the results of the data analysis including EC_{50}s, ICPs, and NOECs; a copy of the reference toxicant control

chart with the concurrent test shown; and a one page summary of compliance with the appropriate QC measures and any deviations (Appendix C).

10.6 The data package will be reviewed by the analyst and the biology team leader or another member of the team before transmittal to the QA officer for final review. The LIMS system will generate a final copy of the results, the case narrative, and a cover memo for transmittal to the end user.

10.7 Analytical Report and Data Package

10.7.1 Analytical reports are produced using the LIMS. The data package is produced from the LIMS and manual log records. Appendix D provides the typical format for data package deliverables.

10.7.2 Include narrative comments regarding sample condition and preservation, calibration and QC performance, and note any significant problems in the LIMS memo field as appropriate.

10.7.3 Document corrective actions taken to meet requirements specified in SOP Section 9 (Quality Control). Where applicable, prepare a laboratory discrepancy form as described in SOP 820 and include the form in the data package.

10.8 SOP Distribution and Acknowledgement

10.8.1 After approval, distribute an electronic copy of the final SOP to all laboratory staff expected to perform the SOP or review data generated by the SOP. The Lab QC Database contains a list of assigned analysts for each SOP. All approved USEPA Region 9 Laboratory SOPs are maintained in the LotusNotes database in portable document format (pdf). Analyst training is documented via the Training Record form and the Read and Understood Signature log; the latter is entered into the Lab QC Database.

10.9 SOP Revisions

10.9.1 Revisions to this SOP are summarized in Appendix R.

11 REFERENCES

EPA Region 9 Laboratory documents (SOPs, the Laboratory Quality Assurance Plan, etc.) are not included in this list. Analysts are referred to the SOP database on Lotus Notes or the local area network (G:\USER\SHARE\QA PROGRAM\LAB SOPS PDF) for these documents; laboratory users should contact the Laboratory QAO for copies of any supporting documents.

Tidepool Scientific Software, 2001. Comprehensive Environmental Toxicity Information

System (CETIS) data analysis/database software application, Version 1.022 User's Guide. Tidepool Scientific Software, McKinleyville, CA.

U.S. EPA, 1995. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms, EPA/660R-95/136.

U.S. EPA, 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, EPA/821/R-02/014.

U.S. EPA, 2010. National Pollution Discharge Elimination System Test of Significant Toxicity Technical Document, EPA/833/R-10/004.

APPENDIX A.
DEVIATIONS FROM THE REFERENCE METHOD

1. No deviations from reference method.

APPENDIX B.
ANALYTES AND TARGET QUANTITATION LIMITS

The following table provides the target analytes list for this SOP with the CAS number and quantitation limits.

Analyte	Chemical Abstracts Registry Number (CASRN)	Water Quantitation Limit, ng/L
Fertilized Eggs Pass (TST)	NA	NA
Fertilized Eggs EC_{50}	NA	NA
Fertilized Eggs NOEC	NA	NA

APPENDIX C.
QUALITY CONTROL MEASURES AND CRITERIA

11.1.1.1

SEA URCHIN AND SAND DOLLAR TOXICITY TEST

Site: _____ Test Date: _____

	YES	NO	COMMENTS
Egg fertilization			
≥ 70% egg fertilization in controls	_____	_____	_____
Dilution water & effluent egg blanks contain no fertilized eggs	_____	_____	_____
NOEC egg fertilization >80% controls	_____	_____	_____
Sperm:Egg Ratio			
Sperm count for final sperm stock ≤ 33,600,000/mL	_____	_____	_____
If sperm count $5.6x10^6$-$33.6x10^6$, actual density ≤ 2 x target density in trial	_____	_____	Target density = _____ Actual density s= _____
If no target density used, high sperm density controls ≥ 5% fertilization than low sperm density controls	_____	_____	_____
Reference Toxicant Test			
Control chart attached	_____	_____	_____
NOEC value _____			_____

NOEC (No Observable Effect Concentration) - The highest concentration of a toxicant at which no adverse effects are observed on the test organisms (i.e., the highest concentration that is not statistically significantly different from the controls).

EC50 _____ Low Confidence Interval _____
High Confidence Interval _____

EC_{50} (50% Effect Concentration) - The EC_{50} is the concentration of a toxicant that would cause a 50% reduction in fertilization for the test population.

% MSD < 25% relative to control	_____	_____	_____

%MSD - A measure of the within-test variability representing the amount of difference from the control that can be detected statistically. (%MSD = MSD/control mean x 100).

Water Quality Measurements (within the acceptable ranges listed below)

Acceptable Range			
Dissolved oxygen (mg/L) ≥4.0mg/L	____	____	_____
pH (units) 7.5-8.5	____	____	_____
Salinity (ppt) 32-36	____	____	_____
Temperature (°C) 11-13	____	____	_____

Name: _____ Title: _____

APPENDIX D.
TYPICAL DATA PACKAGE FORMAT

Data package contents, in order. Optional sections are shown in *italics* text.

Draft Report (from LIMS)

Review Forms
- EPA Review Form
- *Discrepancy Reports (if applicable)*
- *Work Order Memo (if applicable)*

Tracking Forms
- LIMS Work Order(s)
- COC(s)

Analysis Method Data
- Bench sheet(s)
- Raw data sheets

Alternatively, separate calibration and sample data as:
- Sample Data
- Water quality meter calibrations (e.g., pH, DO, refractometer)

Miscellaneous Data
- Other data as applicable

APPENDIX E.
TEST CONDITIONS AND TEST ACCEPTABILTIY CRITERIA

Summary of test conditions and test acceptability criteria for the sea urchin, (*Strongylocentrotus purpuratus*) and sand dollar (*Dendraster excentricus*) fertilization toxicity test.

Test Type:	Static non-renewal
Temperature:	$12^{o}C \pm 1^{o}C$
Salinity:	34 $\pm$ 2 ppt
Light intensity:	10-20 $\mu E/m^2/s$
Test Chamber:	16 x 125 mm test tubes
Test Solution Volume:	5 mL
No. eggs and sperm/test chamber:	1,120 eggs and $\leq 3.36 \times 10^7$ sperm
Number of Replicates:	$\geq$ 4 (10 replicates are recommended for both the control & critical concentration using TST statistical analysis)
Aeration:	Only necessary if $\geq$65% dissolved oxygen saturation cannot be achieved without aeration
Dilution Water:	Uncontaminated at least 1-µm-filtered natural seawater
Test Duration:	40 minutes
Sample volume required:	1L per test
Endpoint:	Fertilization
Test Acceptability:	a) Mean control fertilization $\geq$70% b) Fertilization at NOEC in the reference toxicant $\geq$ 80% of that in the controls c) %MSD <25% in reference toxicant test d) Final sperm stock $\leq$ 33,600,000/mL

APPENDIX F.
WORKSHEET 1: SEA URCHIN FERTILIZATION TOXICITY TEST SPAWNING

CETIS Test No._____ Date _____

No.	Injection time 1	Injection time 2	Spawn Time & Sex	Accepted? (Comments)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				

***Circled numbers indicate test organisms' gametes chosen for the test**

APPENDIX G.
WORKSHEET 2: EGG DENSITY COUNTS

CETIS Test No._____ Date _____

Egg Dilution

eggs counted _____A _____

_____ B _____

Use (A x 10) or (B x 100) _ _____D = # eggs/mL in stock

If the egg stock is > 2,240 eggs/mL (A >224 or B > 30 eggs/mL), dilute the egg stock by transferring:

224,000 eggs/_____ eggs/mL = _____ mL

of well-mixed egg stock to a 100 mL graduated cylinder and bring the total volume to 100 mL with dilution water.

If the egg stock is < 2,240 eggs/mL (A < 224 eggs/mL), concentrate the eggs by allowing them to settle and then decant enough water to retain the following percent of the original volume:

(_____ eggs/mL/ 2,240) x 1000 = % volume

Final Egg Stock

Add 1 mL final egg stock to 9 mL dilution seawater. Count number of eggs.

eggs counted _____

Final egg stock density _____ * 10 = _____ eggs/mL

The egg count should be between 200 and 245 (2,000-2450 eggs/mL). If not, adjust egg stock volume and recheck counts.

APPENDIX H.
WORKSHEET 3: SPECTROPHOTOMETRIC MEASUREMENT OF SPERM CELL DENSITY

CETIS Test No._____ Date: _____

_____

1. Warm up the spectrophotometer.

2. Into a scintillation vial that is tared on a balance, add about 0.05 g of collected sperm using a Pasteur pipet. Note the initial sperm weight (S1). Dilute to about 10 g with sea water and note the final weight (W1). Cap the bottle and mix the contents by inverting several times.

3. Into another tared glass dilution bottle on a top-loading balance, add between 1g to 2g (S2) of the first dilution, make up to about 10g with sea water, and note the final weight (W2). Note: Sperm dilution should be such that the absorbance at 750 nm falls between 0.200-0.600.

4. Read the *absorbance* of the diluted sperm in a 1-cm cell at 750 nm using seawater as a blank.

5. Calculate the sperm cell density of the diluted sperm from the regression equation. To get the density of the collected sperm stock, multiply this value by the dilution factor

S1 = _____

W1 = _____

S2 = _____

W2 = _____

$$\frac{W1 \times W2}{S1 \times S2} = \_\_\_\_\_$$

APPENDIX I.
WORKSHEET 4: DETERMINING SPERM DILUTION FOR 3000:1 OR 500:1 SPERM:EGG RATIO USING THE SPECTROPOTOMETER

CETIS Test No._____ Date _____

1. Count in dilution = [-0.625 + (22.673 *Abs)] x 10^7

   Count in dilution = [-0.625 + (22.673 *_____)] x 10^7 = _____

2. Count in stock sperm = Count in dilution * $\frac{W1 \times W2}{S1 \times S2}$

   Count in stock sperm = Count in dilution * _____ = _____

3. For a 3000:1 S:E ratio, look at Table 1 (Appendix Q) for the number of mL of seawater to add to 0.025 mL concentrated sperm for a 3000:1 S:E ratio based on the #2 count in stock sperm.

   _____ mL to add to 0.025 mL sperm stock for 3000:1 S:E

4. For a 500:1 S:E ratio, look at Table 2 (Appendix Q) for the number of mL of seawater to add to 0.025 mL concentrated sperm for a 500:1 S:E ratio based on the #2 count in stock sperm.

   _____ mL to add to 0.025 mL sperm stock for 500:1 S:E

5. For any other S:E ratio, multiply the value obtained in #3 by the inverse proportion that number is to 3000.

   Table value # mL seawater * 2 = mL for 1500:1 stock

   _____ mL * 2 = _____ mL to add to 0.025 mL sperm stock for 1500:1 S:E

APPENDIX J.
WORKSHEET 5: SPERM COUNT USING MICROSCOPE

CETIS Test No._____ Date _____

Add 0.025 mL sperm to 100 mL 1 % acetic acid. Load sperm onto each side of 2 Neubauer hemacytometers and record counts below:

sperm counted _____

_____

_____

_____

Mean Count _____

$$\text{\# sperm/mL (SPM)} = \frac{(\text{dilution})(4{,}000 \text{ squares/mm}^3)(1{,}000 \text{ mm}^3/\text{cm}^3)(\text{mean count})}{(\text{\# small squares counted})}$$

$$\text{SPM} = \frac{(4001)(4000)(1000)(\quad)}{(\quad)} = \_\_\_\_\_$$

Look up this value in Table 1 or 2 (Appendix Q) and determine the mL of seawater to add to concentrated sperm for S:E of 3000:1 or 500:1:

_____
(X)

Multiply (X) by the fraction of 3000:1 S:E ratio chosen based on the trial. For example, if 1500:1 is the ideal S:E, multiply the 3000:1 value by 2.

mL seawater to add to concentrated sperm based on ideal S:E ratio _____ (X) * _____ (Fraction of 3000) =

APPENDIX K.
WORKSHEET 6: SPERM DENSITY TRIAL

CETIS Test No._____ Date _____

S:E Ratio	% Fertilized	Sperm Count	Actual Sperm/mL	Predicted Sperm/mL
3000:1	_____	_____	_____	3.36×10^7
1288:1	_____	_____	_____	1.4×10^7
550:1	_____	_____	_____	6.17×10^6
234:1	_____	_____	_____	2.62×10^6
100:1	_____	_____	_____	1.12×10^6

**THE S:E RATIO CHOSEN FOR THE DEFINITIVE TEST IS CIRCLED

$$\text{sperm/mL (SPM)} = \frac{(\text{dilution})(\text{count})(\text{hemacytometer conversion})(\text{mm3/mL})}{\#\text{ small squares counted}}$$

Where dilution = 1.1
hemacytometer conversion = 4000
mm3/mL = 1000
small squares counted = see Section 8.11.12

$$\text{SPM} = \frac{(1.1)(\quad)(4{,}000)(1{,}000)}{(\quad)} = \_\_\_\_\_ \text{sperm/mL}$$

****The final sperm concentration must not exceed 2x of the target density from the trial.***

APPENDIX L.
WORKSHEET 7: DEFINITIVE TEST SPEM COUNT USING MICROSCOPE

CETIS Test No._____ Date _____

Add 9.0 mL final sperm stock to 1 mL 10 % acetic acid. Load sperm onto each side of 2 Neubauer hemacytometers and record counts below:

sperm counted _____

_____

_____

_____

Mean Count _____
(X)

$$\text{\# sperm/mL (SPM)} = \frac{(\text{dilution})(4{,}000 \text{ squares/mm}^3)(1{,}000 \text{ mm}^3/\text{cm}^3)(\text{mean count})}{(\text{\# small squares counted})}$$

$$\text{Final SPM (S)} = \frac{(1.11)(4000)(1000)(\quad)}{(\quad)} = \_\_\_\_\_ \quad (S)$$

FINAL S:E RATIO

500:1 S:E = 5.6×10^6 sperm/mL and 2240 eggs/mL

$$\text{Eggs in proportion to sperm (E)} = \frac{(\text{Final egg stock density-Worksheet 2})(0.5 \text{ mL})}{(0.1 \text{ mL sperm stock/test container})}$$

$$\text{Final S:E ratio} = \frac{S}{E} = \_\_\_\_\_ = \_\_\_\_\_$$

If this value is between 500:1 and 3000:1, calculate the difference between the sperm controls.

	%Fertilized	% Not Fertilized	
0.05 mL sperm controls	_____	_____	
	_____	_____	Mean fertilized = _____
0.2 mL sperm controls	_____	_____	
	_____	_____	Mean fertilized = _____

The high sperm density controls (0.2 mL sperm stock) must have at least 5% higher fertilization than the low sperm density controls (0.05 mL sperm stock).

APPENDIX M.
WORKSHEET 8: EFFLUENT & REFERENCE TOXICANT PREPARATION – ECHINODERM FERTILIZATION TOXICITY TEST (MULTIPLE CONCENTRATIONS)

CETIS Test numbers: _____ Site: _____ Test Date: _____

Sample Name_____

EFFLUENT CONCENTRATION (%)	VOLUME EFFLUENT (mL)	TOTAL VOLUME SEAWATER (mL)

Sample Name_____

EFFLUENT CONCENTRATION (%)	VOLUME EFFLUENT (mL)	TOTAL VOLUME SEAWATER (mL)

COPPER CONCENTRATION (µg/L)	VOLUME STOCK COPPER SOLUTION (µL)	TOTAL VOLUME SEAWATER (mL)

Stock solution concentration: _____

APPENDIX N.
WORKSHEET 9: EEFFLUENT & REFERNCE TOXICANT PREPARATION – ECHINODERM FERTILZIATION TOXICITY TEST (TST 2 CONCENTRATIONS)

CETIS Test numbers: ________________ Site: __________________ Test Date: _______

Sample Name______________________________

EFFLUENT CONCENTRATION (%)	VOLUME EFFLUENT (mL)	TOTAL VOLUME SEAWATER (mL)

Sample Name______________________________

EFFLUENT CONCENTRATION (%)	VOLUME EFFLUENT (mL)	TOTAL VOLUME SEAWATER (mL)

Sample Name______________________________

EFFLUENT CONCENTRATION (%)	VOLUME EFFLUENT (mL)	TOTAL VOLUME SEAWATER (mL)

Sample Name______________________________

EFFLUENT CONCENTRATION (%)	VOLUME EFFLUENT (mL)	TOTAL VOLUME SEAWATER (mL)

COPPER CONCENTRATION (µg/L)	VOLUME STOCK COPPER SOLUTION (µL)	TOTAL VOLUME SEAWATER (mL)

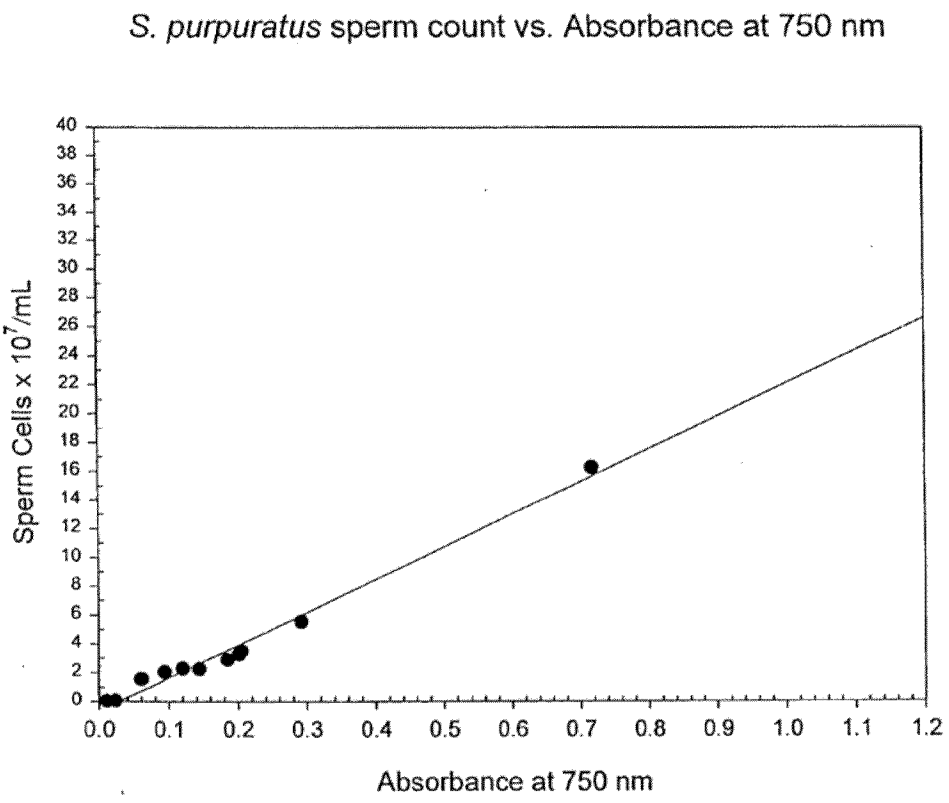
Stock solution concentration: _____________________

APPENDIX O.
WORKSHEET 10: TEST TIMES

CETIS Test No._____ Date _____

	START	FINISH
Sperm added	_____	_____
Eggs Added	_____	_____
Fixative Added	_____	_____

APPENDIX P.
CORRELATION BETWEEN MICROSCOPE COUNTS AND ABSORBANCE READINGS



Linear Regression

Count = -0.625 + (22.673 * Absorbance)

N = 11.000

R = 0.992 Rsqr = 0.984 Adj Rsqr = 0.982

Standard Error of Estimate = 0.591

	Coefficient	Std. Error	t	P
Constant	-0.625	0.252	-2.478	0.035
Absorbance	22.673	0.956	23.714	<0.001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	1	196.633	196.633	562.344	<0.001
Residual	9	3.147	0.350		
Total	10	199.780	19.978		

Normality Test: Passed (P = 0.184)

Constant Variance Test: Passed (P = 0.296)

Power of performed test with alpha = 0.050: 1.000

APPENDIX Q.
DILUTION WATER VOLUMES

TABLE 1 - Dilution water volumes necessary to achieve a sperm stock of 3.36E+7 for a 3000:1 sperm:egg ratio by adding 0.025 mL concentrated sperm.
For example, if the density of the concentrated sperm stock is 3.2E+10, go to the intersection of the first column for 3.00E +10 and to the fourth row for the 0.2 for a volume of dilution seawater 24.32 mL added to 0.025 mL concentrated sperm.

Density	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1E+10	7.44	8.44	9.44	10.44	11.44	12.44	13.44	14.44	15.44	16.44
2E+10	14.88	15.88	16.88	17.88	18.88	19.88	20.88	21.88	22.88	23.88
3E+10	23.32	23.32	24.32	25.32	26.32	27.32	28.32	29.32	30.32	31.32
4E+10	29.76	30.76	31.76	32.76	33.76	34.76	35.76	36.76	37.76	38.76
5E+10	37.2	38.2	39.2	40.2	41.2	42.2	43.2	44.2	45.2	46.2
6E+10	44.64	45.64	46.64	47.64	48.64	49.64	50.64	51.64	52.64	53.64
7E+10	52.08	53.08	54.08	55.08	56.08	57.08	58.08	59.08	60.08	61.08
8E+10	59.52	60.52	61.52	62.52	63.52	64.52	65.52	66.52	67.52	68.52
9E+10	66.96	67.96	68.96	69.96	70.96	71.96	72.96	73.96	74.96	75.96

TABLE 2 - Dilution water volumes necessary to achieve a sperm stock of 5.6E+6 for a 500:1 sperm:egg ratio by adding 0.025 Ml concentrated sperm. For example, if the density of the concentrated sperm stock is 3.2E+10, go to the intersection of the first column +10 and to the fourth row for the 0.2 for a volume of dilution seawater 145.92 mL added to 0.025 mL concentrated sperm.

Density	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1E+10	44.64	50.64	56.64	62.64	68.64	74.64	80.64	86.64	92.64	98.64
2E+10	89.28	95.28	101.28	107.28	113.28	119.28	125.28	131.28	137.28	143.28
3E+10	139.92	139.92	145.92	151.92	157.92	163.92	169.92	175.92	181.92	187.92
4E+10	178.56	184.56	190.56	196.56	202.56	208.56	214.56	220.56	226.56	232.56
5E+10	223.2	229.2	235.2	241.2	247.2	253.2	259.2	265.2	271.2	277.2
6E+10	267.84	273.84	279.84	285.84	291.84	297.84	303.84	309.84	315.84	321.84
7E+10	312.48	318.48	324.48	330.48	336.48	342.48	348.48	354.48	360.48	366.48
8E+10	357.72	363.12	369.12	375.12	381.12	387.12	393.12	399.12	405.12	411.12
9E+10	401.76	407.76	413.76	419.76	425.76	431.76	437.76	443.76	449.76	455.76

APPENDIX R.
REVISION HISTORY

STANDARD OPERATING PROCEDURE: 1001
Revision: 5, Effective: 2/14/14

SEA URCHIN (*Strongylocentrotus purpuratus*) AND SAND DOLLAR (*Dendraster excentricus*) FERTILIZATION TOXICITY TEST

Revision	Effective Date	Description
4	08/31/2010	1. Addition of Section 9.6.3. 2. Inclusion of Appendix P, Revision History. 3. Updated Environmental Management System Section 4.3. 4. Sample storage requirement in Section 5.4 changed to 0-6 °C 5. References updated.
5	02/14/2014	1. Updated Section 2 (addition of TST). 2. Updated Sections 3, 4, 5, 7, 8, 9, 10 and 11, and Appendix D.to current format. 3. Updated Appendix F (TST replicates). 4. Minor edits throughout for clarity and to update requirements of SOP 850 for format. 5. References updated.